Precipio, Inc.

Instructions for Use

KRAS Exon 4 (146) Mutation Analysis using ICE COLD-PCR for Detection with High Resolution Melting

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Manufacturer

This Kit was manufactured by Precipio, Inc. at 8813 F Street, Omaha, NE 68127, USA.
USA Telephone 1-203-787-7888

Reagent Preparation

All reagents supplied with this kit are ready to use. Some components will need to be thawed, vortexed and/ or spun in a microcentrifuge before use; check details in Assay Procedure below. Reagents do need to be combined to produce Master Mixes and reaction mixtures; full details are given in the procedure below.

Kit Components and Storage Conditions

The KRAS Exon 4 (146) Kit contains the components to perform ICE COLD-PCR amplification for mutation enrichment as well as High Resolution Melting for 28 total samples with controls. Recommended storage conditions are listed in Table 1.

Table 1: Kit components with recommended storage conditions.

Reagent, ICE COLD-PCR	Lid Color	Color of Label	Volume Total (µL)	Storage (°C)
Primers/RS-Oligo Mix	Clear	Blue	180	-20
2X Polymerase Master Mix	Clear	Green	900	-20
Wild Type Control	Clear	White	18	-20
1% A146T Control	Clear	Red	18	-20
HRM Reagent	**Lid Color**	**Color of Label**	**Volume Total (µL)**	**Storage (°C)**
HRM Primer Mix, 10 µM	Clear	Orange	40	-20
MeltDoctor™ HRM Master Mix	Black	White	540	-20
Sanger Sequencing Primer, 10 µM*	Clear	Orange	40	-20

*** Can be used for uni-directional Sanger Sequencing confirmation.**

The kit contains enough reagents to perform 32 total analyses. For optimal usage of the kit, it is suggested that 28 samples are run per batch along with one set of controls.

This kit was designed specifically to detect the following mutations:

1. c.436G>T; p.A146T
2. c.436G>C; p.A146P
3. c.436C>T; p.A146V

Reagents Required but not Supplied

1. Molecular Biology Grade Water: Thermo Fisher Catalog # AM9937

Primary Sample Collection, Handling and Storage

This Kit can be used with the following:

- DNA extracted from formalin-fixed paraffin-embedded tumor samples (FFPE slides & blocks) or fine needle aspirations (FNAs)
- Circulating free DNA (cfDNA) from plasma or serum
- DNA isolated from other body fluids

For optimal DNA extraction from FFPE, the tissue should be fixed in neutral buffered formalin for 14–24 hours, placed in ethanol and then embedded in paraffin following standard histological practices. Tumor biopsies are a heterogeneous mixture of tumor cells and non-tumor cells. In addition **the tumor itself is a heterogeneous mixture of tumor cells with mutations and tumor cells without mutations**. Because these somatic mutations may not be evenly distributed throughout the tumor, the resultant mutational analysis of different sections from the same tumor may be different. To increase the probability of detecting a mutation, DNA from the tumor region of the tissue should be isolated by scraping only the tumor area from the glass slide using a fresh, sterile scalpel for each new slide. It is recommended that at least two independent analyses are performed for each sample.

Notes for this Assay

- The ICE COLD-PCR assay has been optimized using the **Bio-Rad C1000** thermal cycler.
- A single sequencing primer is provided for uni-directional sequencing.
- Due to heterogeneity associated with tumors, biopsy samples may contain normal cells as well as Wild-Type and mutant tumor cells.
- The Limit of Detection (LOD) of any mutations present in the sample DNA following ICE COLD-PCR is dependent on the sensitivity of the downstream sequence detection platform used.
- Only the DNA Polymerases supplied with this kit should be used as indicated for the ICE COLD-PCR assays.
- This is a mutation enrichment assay. Any mutation or mismatch covered by the RS-Oligo will be enriched during the ICE COLD-PCR process. There are two possible sources of false positive results related to this assay:
    - Polymerase errors. While the polymerase included in the kit is a high-fidelity enzyme, there is the possibility of a polymerase-induced error.
    - The formalin-fixation process used in preparing FFPE tumor biopsy samples may result in deamination of cytosines. Deamination converts cytosine to uracil. The polymerase will recognize this uracil as a thymine and incorporate an adenine in the copied strands. The correct allele G is then replaced with an A causing an artifact mutation due to the fixation process and not a true somatic mutation.
        - It is highly suggested that an extraction protocol which incorporates Uracil-N-Glycosylase be used for FFPE Extractions.
    - These mutations do not repeat upon re-analysis. Therefore, it is recommended that any such mutation be confirmed by duplicate analysis using the same extracted genomic DNA.

Principles of High Resolution Melting

High Resolution Melting (HRM) is a screening technique which allows the user to determine if the PCR fragment contains a variant or not based upon the dissociation of double stranded DNA in the presence of an intercalator dye as the temperature increases. As the temperature increases, changes in the fluorescence due to the amplicon dissociation are monitored real-time. It is expected that fragments containing a variant will have slightly different fluorescence profiles than that of Wild-Type DNA which allows for the entire amplicon to be screened for mutations.

Since this is a scanning technique and does not identify an exact mutation, any variant present in the PCR product may differentiate from the Wild-Type DNA, thus leading to a mutation positive result. Because HRM is a highly sensitive assay, factors such as pipetting errors, cytosine deamination and polymerase errors may give rise to these false positives. Any positive HRM result must be confirmed by a sequencing methodology or SNP genotyping assay.

ICE COLD-PCR Protocol

IMPORTANT! Use dedicated hood/room for ICE COLD-PCR reaction setup to avoid contamination from post-PCR products.

IMPORTANT! The following procedures are optional but highly recommended prior to PCR setup:

- Turn on UV light inside hood or a UV crosslinker.
- Prior to preparing Master Mixes, UV crosslink all empty Master Mix tubes. Also UV crosslink 1.7 mL tubes containing appropriate volume of Molecular Biology Grade Water needed for Master Mix preparation. These tubes should be UV irradiated for 10 min.
- Make sure all work areas are prepared for analysis of low level mutations. This includes correct use of the PCR Workstation, dedicated pipettes, tips, 10% bleach solution and/or DNA Away™ solutions.

1. Remove PCR Primer/RS-Oligo Mix and 2X Polymerase Master Mix from freezer and thaw on ice.
2. Once thawed, vortex all kit components ~3-5 sec to mix thoroughly. Briefly centrifuge 5 sec to ensure no liquid remains on tube lids and place on ice.

3. **Example experiment layout**: Follow Table 2 for layout to simplify later steps of the procedure. The following 3 controls are required for each setup: **NTC** (No Template Control) and the control DNAs provided by the kits, including **WT** (KRAS wild type) and **A146T_1per** (1% KRAS-A146T).

NOTE: Additional controls may be added in place of test samples.

Table 2: Proposed experimental layout for the ICE COLD-PCR for 32 reactions (28 samples plus controls).

	1	2	3	4
A	SMP01	SMP09	SMP17	SMP25
B	SMP02	SMP10	SMP18	SMP26
C	SMP03	SMP11	SMP19	SMP27
D	SMP04	SMP12	SMP20	SMP28
E	SMP05	SMP13	SMP21	WT
F	SMP06	SMP14	SMP22	A146T_1per
G	SMP07	SMP15	SMP23	NTC1
H	SMP08	SMP16	SMP24	***Leave Empty**

** This well will be used for No Template Control #2 (NTC2) in later steps.*

4. Use Table 3 as a guide for calculating and preparing the ICE COLD-PCR Master Mix assuming a DNA volume of 15 µL at 3.3 ng/µL. Please note that less total DNA can be added to the reaction.
   a. If the extracted DNA concentration is > 3.3 ng/uL, dilute to a working concentration of 3.3 ng/µL and use 15 µL of the diluted DNA for analysis.
   b. If the extracted DNA concentration is ≤3.3 ng/µL, add 15 µL of extracted DNA for analysis.

Table 3: Calculations for Master Mix preparation:

Reagent:	1X reaction*
UV-treated Molecular Biology Grade Water (µL)	5
2X Polymerase Master Mix (µL)	25
Primer/RS-Oligo Mix (µL)	5
Total Volume PCR Master Mix for 1 reaction (µL)	35
Volume DNA added to reaction (µL)	15

**Multiply volumes in this table by the number of samples being tested.*

NOTE: A Master Mix volume slightly greater than this calculation will be required to allow for losses during pipetting.
NOTE: Amount of UV-treated Molecular Biology Grade Water and volume of DNA can be adjusted accordingly.
NOTE: We suggest **fifty** ng of DNA should be used for each PCR reaction in order to potentially detect ≥ 0.1% mutation present in the starting material (minimum 18 ng). The assay has been shown to be robust for DNA input amounts between 1 and 300 ng (using high quality DNA). Up to 20 µL DNA can be used in each PCR reaction with adjustment of volume of water used.
NOTE: If DNA contains contaminants (ethanol carryover, EDTA, etc.), increasing DNA volume may decrease PCR efficiency.

5. Label a 1.7 mL-centrifuge tube for PCR Master Mix preparation.
6. Add required volume of molecular biology grade water to Master Mix tube.
7. Add required volume of 2X Polymerase Master Mix to Master Mix tube.
8. Add required volume of Primer/RS-Oligo Mix to Master Mix tube.
9. Cap Master Mix tube, vortex for ~3-5 sec, briefly centrifuge and store on ice until use.
10. Label a 96-well plate with appropriate sample information.
11. Pipette appropriate volume of ICE COLD-PCR Master Mix into applicable wells. If using a repeat pipettor, ensure that there is no spillage or splashing from well to well.
12. To appropriate wells, add required volume of each DNA sample, control, or water (NTC). Use separate pipette tips for each sample and avoid cross-contamination of the samples by splashing. Securely cap all wells containing DNA samples, controls, and NTC with 8-cap strips.

    NOTE: Addition of the kit controls last lessens the chance of contaminating any test sample wells.

13. Vortex (1/2 speed) for ~3-5 sec. Centrifuge for ~5 sec to ensure all solutions are collected at the bottom of wells or tubes. If not, repeat centrifugation.

Thermal Cycler Program for ICE COLD-PCR

Use the thermal cycler protocol in Table 4 for ICE COLD-PCR.

IMPORTANT! Ensure that the following ramp rates are used depending on the thermal cycler:
- BioRad C1000 Touch: 1.5°C/sec
- BioRad Tetrad II: default (3.0°C/sec)
- Applied Biosystems Veriti: 100%

Table 4: Thermal cycler conditions for ICE COLD-PCR.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 sec	1
Main ICE COLD-PCR Amplification	98 °C	10 sec	45
	69 °C	30 sec	
	73.5 °C	30 sec	
	63 °C	30 sec	
	72 °C	20 sec	
Amplification	98 °C	10 sec	5
	63 °C	10 sec	
	72 °C	20 sec	
Final Extension	72 °C	5 min	1
Hold	12 °C	∞	

NOTE: Samples can be stored at -20 °C post amplification.

Quality Control of ICE COLD-PCR Products (OPTIONAL)

1. Analyze an aliquot of ICE COLD-PCR product alongside an aliquot of 100-bp DNA mass ladder on a 2% agarose gel to verify amplification of DNA.
2. Only a single band corresponding to the main ICE COLD-PCR product should be observed (~125-170 bp).
3. If multiple smaller bands (<80 bp) are present check quality of input DNA.
4. If no product is observed, ensure quality of input template DNA was sufficient.
5. No ICE COLD-PCR products should be visible in No Template Control sample.
    a. If DNA products of the expected product size are visible in No Template Control sample, contamination is likely and sample should not be taken to sequence analysis.

Downstream Analysis by High Resolution Melting (HRM)

1. For HRM, please see universal HRM IFU for analysis.

Technical Support/Questions

For any questions regarding the kit or IFU, please contact us at Techsupport@precipiodx.com or call 1.203.787.788 ext. 509.